Effects of heme proteins on nitric oxide levels and cell viability in isolated PMNs: a mechanism of toxicity

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Abstract: Isolated human PMNs served as a model to determine oxyhemoglobin (oxyHb) binding and the effects of oxymyoglobin (oxyMb) or oxyHb on production of both nitric oxide (NO·) and superoxide ($O_2 \cdot^-$) and the resulting cytotoxicity. Physiologically relevant concentrations of NO· and H_2O_2 oxidized, to a similar extent, 2,7-dichlorodihydrofluorescein (DCFH) loaded into polymorphonuclear neutrophils (PMNs). Activation of PMNs with phorbol 12-myristate 13-acetate (PMA) markedly increased the internalization of extracellular oxyHb (10–250 µg/mL). OxyMb (10–300 µg/mL) or oxyHb (30–300 µg/mL) enhanced DCFH oxidation by a concentration-dependent mechanism in unstimulated, lipopolysaccharide (LPS) and tumor necrosis factor α (TNF-α)-, and PMA-stimulated PMNs. This increased DCFH oxidation was eliminated by NO· synthase inhibitors, glutathione and ascorbate, and was reduced by albumin. Nitrite accumulation in PMN filtrates mirrored NO·-induced DCF fluorescence. OxyMb-induced increases in NO· levels paralleled alterations in DNA and cell membrane damage and ATP levels in PMNs and co-cultured lymphocytes, and were attenuated by NO· synthase inhibitors. OxyMb eliminated extracellular $O_2 \cdot^-$ at protein concentrations 100- to 1000-fold above those of superoxide dismutase. These results suggest that heme proteins bind and internalize into PMNs and increase NO·-induced damage in neighboring cells by inhibiting $O_2 \cdot^-$-scavenging of NO·. We propose a mechanism whereby heme protein-induced NO· levels may contribute to immunosuppression and increased infection rates associated with transfusions and cellular damage during inflammation. *J. Leukoc. Biol.* 67: 357–368.

Key Words: *apoptosis · superoxide · myoglobin · hemoglobin · DNA strand breaks · cytotoxicity*

INTRODUCTION

Severe tissue injury, rhabdomyolysis, and myocardial infarction result in the release of myoglobin (Mb) from muscle into the interstitium and the vasculature [1–6]. Hemoglobin (Hb) is released from erythrocytes during hemolytic disorders, burns, and storage of blood for transfusion [7–10]. In humans, elevated levels of Mb or Hb have been reported to be associated with acute renal failure [11, 12], infections, and recurrent cancers in postoperative patients receiving autologous and/or allogeneic transfused blood [13, 14]. A recent report of higher mortality in trauma patients transfused with acellular, cross-linked oxyhemoglobin (oxyHb) solutions instead of stored red blood cells (RBCs) emphasizes the importance of elucidating the mechanism of heme protein toxicity [15].

The addition of Hb or Mb to experimental models of bacterial peritonitis [16] or glomerulonephritis [17] resulted in decreased survival and a reduction of white blood cell count in the rat. OxyHb added to phorbol 12-myristate 13-acetate (PMA)-stimulated polymorphonuclear neutrophils (PMNs) reduces PMN viability, production of $O_2 \cdot^-$, and bacterial killing capability [18]. OxyHb has also been reported to augment the production of nitric oxide (NO·) in interleukin-1 (IL-1)-induced cultured smooth muscle cells [19]. Decreasing NO· levels by selectively inhibiting the inducible nitric oxide synthase (iNOS) prolongs survival time in the bacterial peritonitis model [16]. In addition to its cytotoxic effects, NO· has recently been demonstrated to act as an early proinflammatory mediator by increasing inflammatory transcription factors and cytokine expression [20]. Decreased survival in these infection models induced by heme proteins may be due to a reduced scavenging of NO· by $O_2 \cdot^-$, increasing NO· extracellular levels that damage neighboring cells.

We propose a mechanism whereby heme proteins, known to bind to NO·, may actually increase extracelluar NO· levels derived from activated PMNs at certain heme protein concentrations. PMA activation of PMNs increases binding and internalization of oxyHb into PMNs [18] and increases the production of $O_2 \cdot^-$ and NO·. The levels of $O_2 \cdot^-$ produced by PMA-activated PMNs have been reported to exceed NO· levels by twofold [21]. Superoxide reacts rapidly with NO· to form peroxynitrite (ONOO$^-$), with rates of $37 \times 10^6 \cdot M^{-1} \cdot s^{-1}$ [22] and more slowly with both oxyHb and metHb to form metHb and oxyHb and H_2O_2 and O_2, respectively [23]. Peroxynitrite is a larger, charged compound that should not enter neighboring cells as readily as NO·. It is conceivable that the binding and internalization of heme proteins into PMNs may reduce $O_2 \cdot^-$ levels [18] to a greater extent than NO· [23].

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Heme protein augmentation of NO$\cdot$ levels may occur in cells that produce both NO$\cdot$ and $O_2\cdot^-$. Nitric oxide synthase (NOS) has been found in neutrophils, endothelial cells, macrophages, hepatocytes, Kupffer cells, smooth muscle cells, fibroblasts, and renal epithelial cells [25, 26]. NADPH oxidase, producing varying levels of $O_2\cdot^-$, has been characterized in these same cells [27–31]. Our hypothesis is that in activated PMNs heme proteins bind to the membrane and internalize and dismute $O_2\cdot^-$ to H_2O_2 in a superoxide dismutase (SOD)-like manner. This decreases extracellular $O_2\cdot^-$, allowing NO$\cdot$ concentrations to rise, enter neighboring cells, and produce toxic effects.

MATERIALS AND METHODS

PMNs and lymphocyte isolation

All reagents, unless otherwise specified, were obtained from Sigma Chemical Co. (St. Louis, MO). Human PMNs and lymphocytes were obtained from EDTA-preserved venous blood of nonsmoking adult males by layering blood over Polymorphprep, Nycomed Pharma As (Oslo, Norway). Blood was centrifuged at 550 g for 30 min at 22°C, and the PMN and lymphocyte layers were collected and washed twice in Hanks' balanced salt solution (HBSS; GIBCO, Grand Island, NY). All PMNs were incubated with physiological levels (10^{-4} M) of arginine unless otherwise stated. One or two hypotonic lyses were performed to lower the RBCs to $\leq 1\%$ of PMNs or of lymphocytes. RBC ghosts lying on top of the PMNs were removed by pipette extraction after the first hypotonic lyse. The purity of PMNs and lymphocytes was greater than 88 and 95%, respectively, and their viability as determined by trypan blue uptake was greater than 90%. All experiments were conducted with 300,000 PMNs added to 100 µL of HBSS in tubes (12×75 mm) opened to room air unless otherwise stated. Replicate experiments were conducted using PMNs isolated from different donors.

Effect of NO$\cdot$ or H_2O_2 concentration or PMN density on 2,7-dichlorodihydrofluorescein (DCFH) oxidation

PMNs were incubated with DCFH-diacetate (DCFH-DA; 2 µM; from Kodak, Rochester, NY) at 37°C for 15 min. DCFH-DA permeates cells freely and is trapped after enzymatic hydrolysis of the diacetate to DCFH. Oxidation of DCFH results in the fluorescent DCF. To assess the use of DCFH-DA in measuring intracellular levels of NO$\cdot$ and/or H_2O_2, a NO$\cdot$ donor, diethylamine nitric oxide (DEANO; Molecular Probes, Eugene, OR) or H_2O_2, at concentrations of 10^{-11} to 5×10^{-4} M were incubated with DCFH-loaded PMNs at 37°C for 30 min. Concentrations of DEANO-derived NO$\cdot$ were generated by rapidly performing an initial10-fold serial dilution of a concentrated (10^{-2} M) alkali (1 mM NaOH) stock into HBSS buffer followed by a 10-fold dilution into the PMN preparations [32]. Intracellular levels of propidium iodide (PI) were used as a measure of cell membrane integrity. PI staining of oxidant-treated PMNs (as described above) was determined by flow cytometry analyses after a 2- to 3-min treatment with PI (0.75 mM). The effect of density of PMNs on DCFH oxidation was assessed by incubating increasing numbers of PMNs (50,000–1,000,000/ 100 µL HBSS) with PMA (200 nM) for 30 min. Flow cytometric analyses were performed with an argon laser (488 nm) and emission light measured behind a filter transmitting 530/30-nm light on a FACScan, Becton Dickinson (San Jose, CA) with CELLQuest data acquisition and analysis software. For each sample, 10,000 PMNs were collected. The mean channel fluorescence was determined on a linear scale from a single-parameter histogram. Mean channel fluorescence was presented as molecules of equivalent fluorescein using a standard curve prepared from fluorescein microbeads (Flow Cytometry Standards Corp., San Juan, PR).

Heme protein effect on DCFH oxidation in unstimulated, lipopolysaccharide (LPS) and tumor necrosis factor α (TNF-α)-, and PMA-stimulated PMNs

DCFH-loaded PMNs were incubated with and without N-methyl-L-arginine (L-NMMA; 5 mM; Calbiochem, San Diego, CA) [33–35] and heme proteins (0.3–1000 µg/mL) at 37°C for 10 min. The L-NMMA-treated PMNs were subsequently incubated for an additional 30 min in the absence or presence of LPS (10 ng/mL), LPS (10 ng/mL) and TNF-α (1 ng/mL), or PMA (200 nM). In unstimulated and LPS and TNF-α-stimulated PMNs, the treatments were oxyMb, or oxyMb + L-NMMA (5 mM). In PMA-stimulated PMNs with arginine, the treatments were oxyMb, oxyHb, bilirubin, ferrous chloride, or either isolated RBCs or sonicated RBCs containing comparable amounts of oxyHb. RBC membranes were ruptured using a micro-ultrasonic cell disrupter (Kontes, Janke and Kukel, Gmbh and Co., Stauten, Germany). In PMA-stimulated PMNs without arginine, the treatments were oxyMb (dithionite-treated), oxyMb, chromatographically purified oxyHb-A_o (98% ferrous), α-α diaspirin cross-linked oxyHb (98.6% ferrous), oxyHb, or metHb. OxyMb was prepared by mixing excess sodium dithionite with Mb for 1 h [36] and inorganic sulfur compounds were removed by dialyzing in HBSS buffer for 3 h. Percent iron in the ferric state in non-dithionite-treated oxyMb or oxyHb was not assessed. Endotoxin level in α-α Hb as determined by *Limulus* amebocyte lysate assay was 0.125 EU/mL. Diaspirin cross-linked Hb was a gift from Walter Reed Army Institute of Research. To assess whether heme proteins quench DCF fluorescence, PMNs were loaded with oxidized DCF (2 µM; Kodak) and incubated with varying myoglobin concentrations (1–3,000 µg/mL). Flow cytometric measurements of oxidant-induced DCF fluorescence were performed as described above.

OxyMb effects on NO_2^- and NO_3^- levels

Total nitrate and nitrite levels in filtrates of PMNs were determined by reducing the nitrate to nitrite by nitrate reductase and reacting the resulting nitrite with 2,3-diaminonaphthalene to form a fluorescent product [37]. PMNs (500,000/ 300 µL HBSS) were treated with oxyMb (1–1000 µg/mL) and SOD (30 U) for 10 min, followed by PMA (200 nM) for 1 h at 30°C. Filtrates were collected by centrifugation at 16,000 g for 15 min through Ultrafree-MC 10,000 NMWL filters (Millipore, Bedford, MA). Total nitrite levels were determined with a 96-well fluorescence analyzer (IDEXX, Westbrook, ME). PMNs exposed to the same treatment were analyzed by flow cytometry for DCFH oxidation.

OxyHb bound to and internalized into PMNs

OxyHb bound to and internalized into PMNs was determined using fluorescein-conjugated oxyHb (Molecular Probes, Eugene, OR). Each molecule of chromatographically purified oxyHb-A_o (98% ferrous) had an average of 4.5 molecules of fluorescein-5-EX succinimidyl ester conjugated to lysine (oxyHb-FLEX). OxyHb-FLEX (1–250 µg/mL) was incubated with PMNs with and without albumin (20 mg/mL) for 10 min in a shaking water bath at 37°C followed by a 30-min incubation with or without LPS (10 ng/mL) and TNF-α (1 ng/mL) or PMA (200 nM). Flow cytometric measurements were performed as described above. To visualize intracellular levels of oxyHb-FLEX, whole-cell and Z-plane images of PMNs were collected using an Axiovert 135 inverted confocal microscope (Zeiss, Thornwood, NY and Atto Instruments, Rockville, MD).

OxyMb effects on DCFH oxidation in PMA-activated PMNs with and without NO inhibitors, Cu,Zn SOD, or antioxidants

DCFH-loaded PMNs were incubated for 10 min at 37°C with various amounts of oxyMb (1–1000 µg/mL) with or without L-NMMA (5 mM), N-(1-iminoethyl)-L-ornithine (L-NIO; 5 mM), ascorbate (25 µM) and glutathione (25 µM), human albumin (20 mg/mL), or human erythrocyte Cu,Zn SOD (300 U/mL; 80 µg/mL) before a 30-min incubation with PMA (200 nM). Also, various concentrations of bovine liver Cu,Zn SOD (30–10,000 U/mL; 8–2640 µg/mL), and 30 µg/mL oxyMb were incubated with PMA-stimulated PMNs pretreated with and without L-NMMA. Flow cytometric measurements of oxidant-induced DCF fluorescence were performed as described above.

Comparison of oxyMb and SOD effect on extracellular O$_2$$^{\cdot-}$ levels

PMNs (200,000/200 µL HBSS) were treated with various concentrations of oxyMb or bovine liver Cu,Zn SOD (0.05–1000 µg/mL) in a 340 ATTC 96-well reader (SLT-LabInstruments, Salzburg, Austria) and activated with PMA. O$_2$$^{\cdot-}$ production was determined by measuring reduction of cytochrome c at 550 nm.

Phosphatidylserine externalization on plasma membrane of PMNs

Levels of phosphatidylserine on the outer surface of plasma membrane were determined by fluorescein isothiocyanate (FITC)-labeled annexin V (8 µg/300,000 PMNs/100 µL) according to the manufacturer's instructions (Caltag Laboratories, Burlingame, CA). PMNs were incubated with and without L-NMMA (5 mM) and oxyMb (1–1000 µg/mL) for 10 min at 37°C, followed by PMA (200 nM) for 1 h at 37°C. PMNs were then incubated with annexin V for 30 min and PI (0.75 mM) for 2–3 min at 4°C before flow cytometric analyses.

DNA strand breaks

OxyMb effect on DNA strand breaks in PMA-activated PMNs was measured by flow cytometry using the tdt-mediated dUTP nick end labeling assay (*In Situ* Cell Death Detection Kit, Fluorescein, Boehringer Mannheim, Indianapolis, IN) according to manufacturer's instructions. PMNs were incubated with and without L-NMMA (5 mM) and oxyMb (1–1000 µg/mL) for 10 min at 37°C. PMNs were subsequently treated with PMA (200 nM) for 2 h at 37°C. Label solutions without terminal transferase or with DNase I (10 U) served as negative and positive controls, respectively.

Cell membrane integrity and lymphocyte viability

Intracellular levels of PI were used as a measure of cell membrane integrity. PMNs were incubated with and without L-NMMA (5 mM) and oxyMb (1–1000 µg/mL) for 10 min at 37°C followed by PMA (200 nM) for 2 h at 37°C. Membrane integrity was determined by flow cytometry analyses after a 2- to 3-min treatment with PI (0.75 mM).

ATP levels in PMNs

OxyMb effect on ATP levels in PMA-activated PMNs was measured using the Bioluminescent Somatic Cell Assay Kit according to the manufacturer's instructions. PMNs were incubated with and without L-NIO (5 mM) and oxyMb (1–1000 µg/mL) for 10 min at 37°C. PMNs were subsequently treated with PMA (200 nM) for 2 h at 37°C. PMNs were lysed, frozen at −20°C, and analyzed within 24 h. Emitted light was determined in a camera luminometer (Tropix, Redford, MA) using Panchromatic black and white, ISO 20,000, type 612 film (Polaroid; Cambridge, MA) with an exposure time of 2.5 min. Level of exposure was measured and digitized with a solid-state camera (COHU, San Diego, CA) and analyzed using the public domain NIH Image 1.55 program.

Statistical analysis

All values are presented as means ± SEM. The Kolmogorov-Smirnov test was used to verify the normal distribution of the data. Groups were compared by using analysis of variance with the Fisher least-significant difference *post hoc* procedure. Statistical significance was accepted at P values < 0.05.

RESULTS

DCFH as an indicator for measuring oxidants in PMNs

Increasing molar concentrations of DEANO-derived NO$^\cdot$ and H$_2$O$_2$ (10^{-11}–10^{-5} M) incrementally increased DCFH oxidation to similar levels, increasing 25-fold over control levels at 10^{-5} M. However, the 10^{-4} M concentration of both oxidants resulted in an approximately 25% decrease (**Fig. 1A**). Both NO$^\cdot$ and
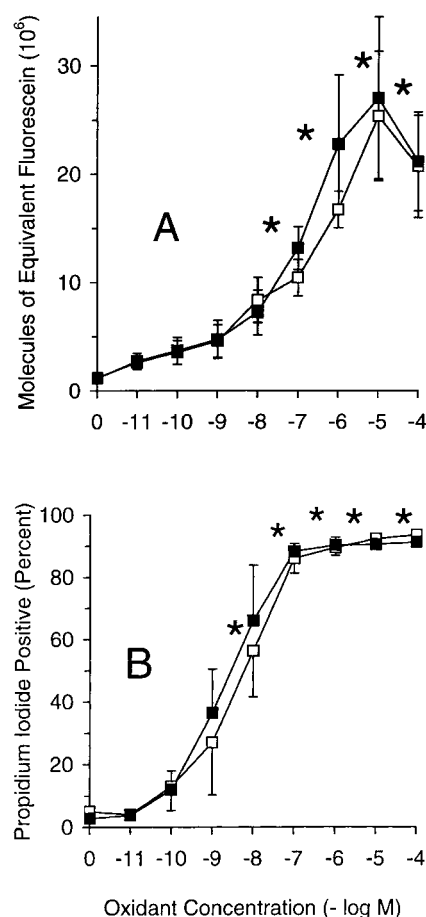

Fig. 1. Concentration-dependent effects of H$_2$O$_2$ (□) or DEANO-derived NO ($\blacksquare$) on (A) DCFH oxidation and (B) PI intracellular staining in PMNs. Data represent means ± SEM of four experiments. Control vs. concentrations of H$_2$O$_2$ or DEANO, *P < 0.05.

H$_2$O$_2$ also incrementally increased cellular membrane damage with 87% of PMNs staining positive for PI at levels ≥10^{-7} M (Fig. 1B). Loss of cellular membrane integrity with leakage of DCF from PMN may account for the loss of fluorescence at the 10^{-4} M concentration of oxidants. PMA-activated PMNs showed an approximate fivefold incremental increase in oxidant-induced DCF fluorescence as the number of PMNs increased from 50,000 to 500,000 in a fixed volume of buffer (**Fig. 2**).

Heme protein effects on DCFH oxidation (NO$^\cdot$) and nitrite levels

None of the oxyMb concentrations (0–3000 µg/mL) used in this study quenched DCF fluorescence in PMNs (data not shown). OxyMb at concentrations of 1–1000 µg/mL incubated with unstimulated, LPS and TNF-α-, and PMA-stimulated PMNs induced a concentration-dependent, biphasic response in DCFH oxidation. In unstimulated PMNs, 30 µg/mL of oxyMb induced an approximately fourfold increase in DCFH oxidation compared with the 0 and 1000 µg/mL oxyMb concentrations (**Fig. 3**). L-NMMA, a competitive inhibitor of NOS, lowered the oxyMb (30 µg/mL)-induced increase in DCFH oxidation by 50%. In LPS and TNF-α-stimulated PMNs, DCFH oxidation peaked at 30 µg/mL of oxyMb with an approximately six- and threefold increase in DCFH oxidation compared, respectively,
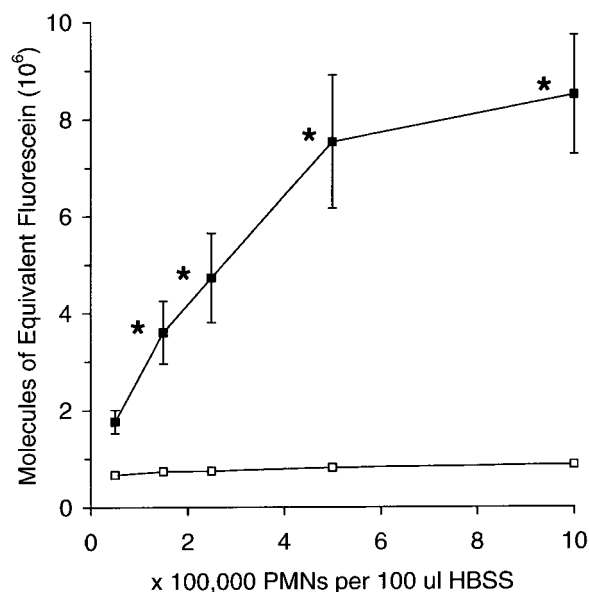
Fig. 2. Effect of PMN density (PMNs/100 μL buffer) on DCFH oxidation as determined in the presence (■) or absence (□) of PMA. Data represent means ± SEM of three experiments. Preparation of PMNs (50,000) vs. PMNs (150,000–1,000,000), *P < 0.05.

with the 0 and 1000 μg/mL oxyMb concentrations (Fig. 3). Again, L-NMMA lowered the oxyMb (30 μg/mL)-induced increase in DCFH oxidation by 38%.

In PMA-stimulated PMNs, oxyMb produced a concentration-dependent increase in DCFH oxidation, with the peak response at 30 μg/mL. This level of oxyMb increased DCFH oxidation approximately threefold compared with the 0 and 1000 μg/mL oxyMb concentrations (**Fig. 4A**). Oxidant levels in PMA-stimulated PMNs treated with 0 and 30 μg/mL oxyMb were six-
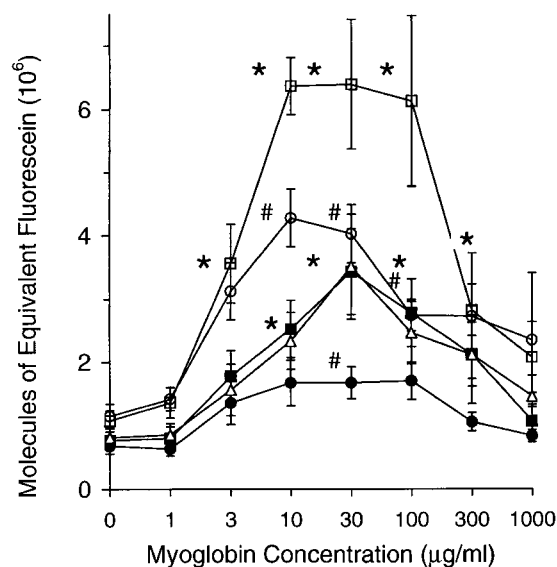

Fig. 3. Concentration-dependent effect of oxyMb on DCFH oxidation in unstimulated (n = 7; ■), unstimulated + L-NMMA (n = 7; ●), LPS-stimulated (n = 6; △), LPS and TNF-α-stimulated (n = 7; □), LPS and TNF-α-stimulated + L-NMMA PMNs (n = 5; ○). Data represent means ± SEM of five to seven experiments. Control vs. concentrations of oxyMb for each treatment condition, *P < 0.05; unstimulated or LPS and TNF-α-stimulated PMNs with vs. without L-NMMA, #P < 0.05.
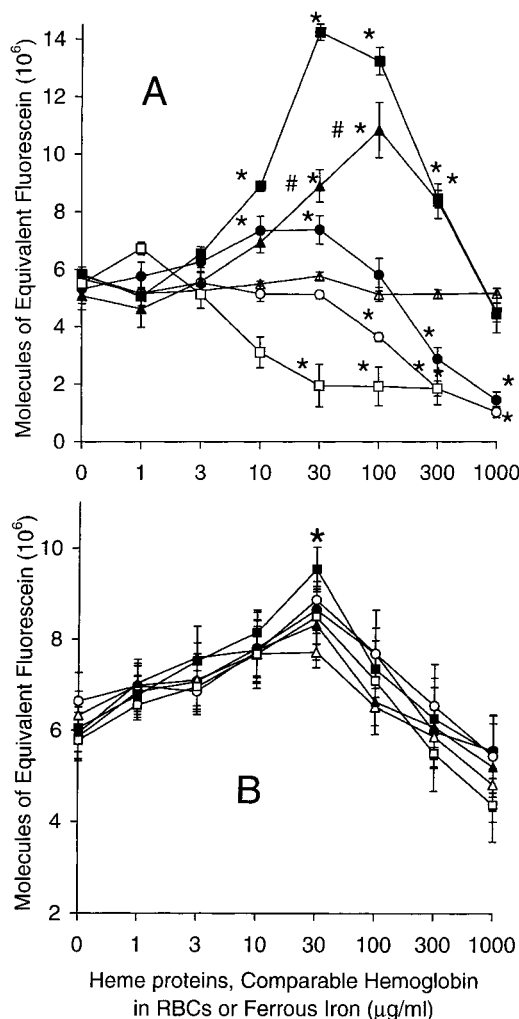

Fig. 4. Concentration-dependent effects of heme proteins, RBCs, or ferrous iron on DCFH oxidation in PMA-stimulated PMNs with (A) and without (B) arginine. (A) Arginine-loaded PMNs were treated with varying concentrations of oxyMb (■), oxyHb (▲), RBCs (○), sonicated RBCs (●), bilirubin (△), or ferrous iron (□). (B) PMNs without arginine were treated with varying concentrations of oxyMb (■), mixed oxyMb and metMb (□), oxyHb-A₀ (○), α-α diaspirin cross-linked oxyHb (▲), mixed oxyHb and metHb (●), or metHb (△). Data represent means ± SEM of four experiments. Control vs. concentrations of heme proteins, RBCs, or iron, *P < 0.05; oxyMb vs. comparable oxyHb concentrations, #P < 0.05.

and twofold higher than those of LPS and TNF-α-stimulated PMNs, respectively (Fig. 4 vs. Fig. 3). The oxyHb effect on DCFH oxidation paralleled oxyMb except that oxyHb was approximately 20–40% less efficient in inducing DCFH oxidation. RBC preparations containing comparable amounts of oxyHb as above did not increase DCFH oxidation, but did produce an incremental decrease in DCFH oxidation at RBC concentrations of oxyHb ≥100 μg/mL. When comparable concentrations of RBCs were sonicated, DCFH oxidation was intermediate between oxyHb and intact RBCs, producing higher DCFH oxidation than intact RBC at 10, 30, and 100 μg/mL and DCFH oxidation comparable to that of intact RBC at 300 and 1000 μg/mL. Thus, the encapsulation of oxyHb in the RBC membranes appears to have reduced the DCFH oxidation levels. Bilirubin, the non-protein breakdown product of hemoglobin, did not affect DCFH oxidation. Addition of $FeCl_2 \cdot 4H_2O$

produced a decrease in DCFH oxidation above 30 μg/mL. On a molar basis, the Fe^{2+} in 1 μg/mL of $FeCl_2 \cdot 4H_2O$ equates to 85 μg/mL oxyMb. Iron-induced decreases in DCFH oxidation coincided with decreases in PMN viability (data not shown).

PMA-stimulated PMNs were also treated with oxyMb (pretreated with dithionite), oxyMb, chromatographically purified oxyHb-A_0 (98% ferrous), α-α diaspirin cross-linked oxyHb (98.6% ferrous), oxyHb, or metHb in HBSS without arginine (Fig. 4B). All heme proteins except metHb produced similar DCFH oxidation profiles, inducing an increase in DCFH oxidation at the 30 μg/mL concentration. Removing arginine from the preparation decreased the heme protein-induced DCFH response by 25–50% (Fig. 4, A vs. B). Levels of nitrite, a stable product of oxidized NO·, in the PMN filtrates paralleled the profile of DCFH oxidation (**Fig. 5**). OxyMb (30 μg/mL)-treated PMNs produced a doubling of nitrite levels compared with untreated PMNs.

OxyHb bound to and internalized into PMNs

OxyHb-FLEX binding to unstimulated, LPS and TNF-α-stimulated (100 and 250 μg/mL), and PMA-stimulated PMNs (30, 100, and 250 μg/mL) increased incrementally with oxyHb-FLEX concentration (**Fig. 6**). OxyHb-FLEX bound to and internalized into PMA-stimulated PMNs was two- to sixfold times that of unstimulated PMNs at concentrations ≥30 μg/mL. Pretreating PMA-activated PMNs with human albumin at levels found in extracellular spaces of tissues (330 μM; 20 mg/mL) did not alter oxyHb binding (data not shown). Whole PMN and Z-plan fluorescent images (0.4 μM) of PMA-activated PMN treated with oxyHb-FLEX (250 μg/mL) showed clusters of localized oxyHb-FLEX throughout the cytosol (**Fig. 7**).

Effects of NO inhibitors and antioxidants on DCFH oxidation

As with unstimulated and LPS- and TNF-α-stimulated PMNs, L-NMMA eliminated oxyMb-induced increases in oxidant pro-
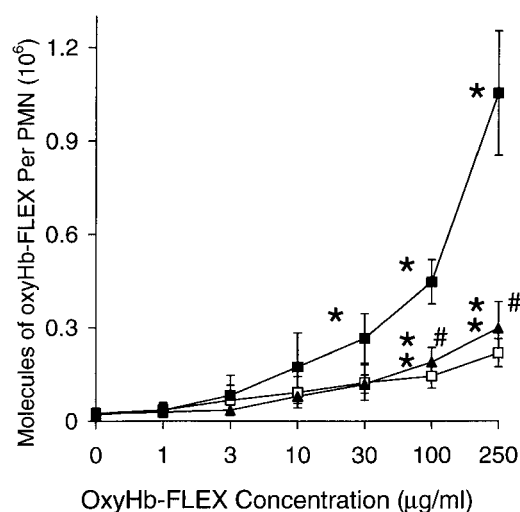
Fig. 6. OxyHb binding to and internalization into stimulated and unstimulated PMNs. Binding was assessed by using OxyHb-conjugated fluorescein-EX (4.5 molecules/oxyHb). OxyHb-FLEX (□), oxyHb-FLEX + LPS and TNF-α (▲) and oxyHb-FLEX + PMA (■). Data represent means ± SEM of four to five experiments. Control vs. concentrations of oxyHb-FLEX bound to and internalized into unstimulated or stimulated PMNs, *$P < 0.05$; oxyHb-FLEX vs. comparable oxyHb-FLEX + PMA concentrations, #$P < 0.05$.

duction in PMA-stimulated PMNs (**Fig. 8A**). L-NIO, a noncompetitive inhibitor of NOS, mirrored the effect of L-NMMA. Physiological levels of glutathione (25 μM) and ascorbate (25 μM) further reduced DCFH oxidation levels below L-NMMA- and L-NIO-inhibited levels, suggesting that a part of the DCFH oxidation is due to H_2O_2 or other oxidants besides NO· or that inhibition of NO synthase was incomplete. Human albumin at levels found in the extracellular space of tissues also reduced oxyMb-induced DCH fluorescence by approximately 75%. This level of albumin (20 mg/mL), which is 20-fold greater than the highest heme protein concentration (1 mg/mL), may have partially reduced DCF fluorescence by sequestering PMA from activating PMNs. However, in contrast to glutathione and ascorbate, oxyMb (100 μg/mL) in the presence of albumin still induced DCFH fluorescence at five times control levels (data not shown).

The superoxide scavenger, human erythrocyte Cu,Zn SOD (300 U/mL, 80 μg/mL), increased oxyMb induced-DCFH oxidation (Fig. 8A). Bovine liver SOD (30–10,000 U/mL) produced a biphasic, concentration-dependent increase in DCFH oxidation, with 300 U/mL SOD producing peak levels in the presence of 30 μg/mL of oxyMb (Fig. 8B). This concentration of oxyMb was selected because it consistently generated peak levels of DCF fluorescence when used alone (Figs. 3, 4, and 8A). At SOD concentrations ≤600 U/mL, L-NMMA eliminated the SOD-induced increases in DCF fluorescence. The highest concentrations of bovine liver SOD (10,000 U/mL; 26,300 μg/mL) reduced DCF fluorescence below control levels. Bovine liver SOD and oxyMb both exhibited a concentration-dependent reduction of extracellular $O_2^{\cdot-}$ produced by PMA-stimulated PMNs, with SOD being 100- to 1,000-fold more potent (**Fig. 9**).
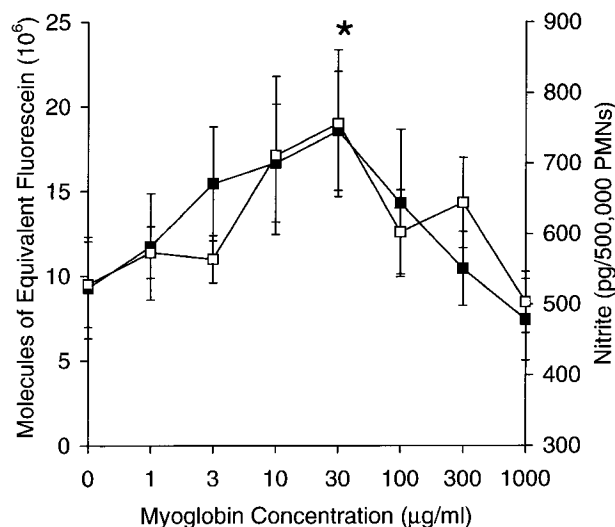

Fig. 5. Concentration-dependent effects of oxyMb + 100 U/mL Cu,Zn SOD on DCFH oxidation (■) and nitrite levels (□) in PMA-stimulated PMNs. Data represent means ± SEM of five or seven experiments, respectively. Control vs. concentrations of oxyMb on DCF fluorescence and nitrite levels in filtrates in PMNs, *$P < 0.05$.
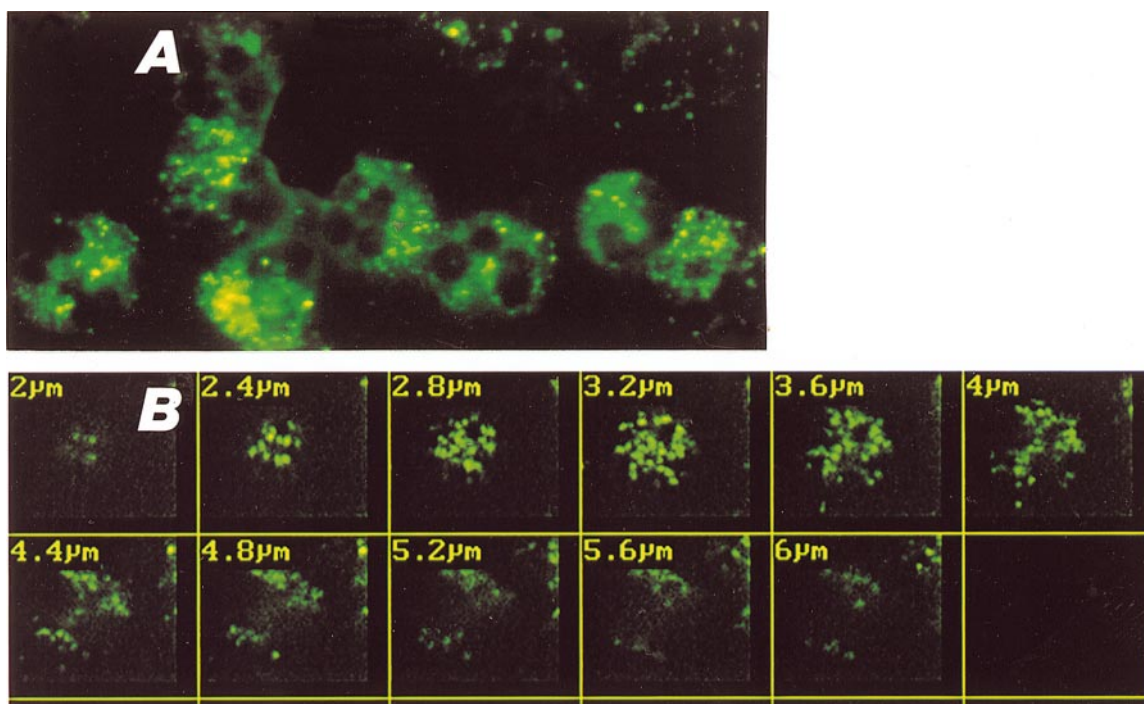
Fig. 7. Bound and internalized oxyHb-FLEX fluorescence in PMA-activated PMNs. (A) Whole-cell and (B) Z-plane confocal images of PMNs incubated with oxyHb-FLEX (250 μg/mL).

NO· effects on phosphatidylserine externalization on plasma membrane, DNA strand breaks, plasma membrane integrity, and ATP levels in PMA-stimulated PMNs and lymphocytes

In each of these endpoints, the oxyMb-induced incremental changes in NO· levels paralleled incremental changes in phosphatidylserine externalization on cell membranes (**Fig. 10A**), DNA strand breaks (Fig. 10B), plasma membrane integrity (Fig. 10C), and intracellular ATP levels (Fig. 10D). Phosphatidylserine-positive PMNs were fivefold (20 vs 3.7%) greater in PMNs treated with oxyMb (30 μg/mL) compared with PMNs without oxyMb. L-NMMA eliminated 68% of the oxyMb (30 μg/mL)-induced increases in phosphatidylserine levels on plasma membranes of PMNs. PMNs containing DNA strand breaks were fourfold (50 vs. 12%) greater in PMNs treated with oxyMb (30 μg/mL) compared with PMNs without oxyMb. L-NMMA reduced the oxyMb (30 μg/mL)-induced increase in DNA strand breaks by 30%. PMN plasma membrane damage (i.e., reduced viability) was threefold (87 vs. 32%) higher in PMNs treated with oxyMb (30 μg/mL) compared with PMNs without oxyMb. L-NMMA eliminated 97% of the oxyMb (30 μg/mL)-induced decreases in PMN viability. Intracellular ATP levels were ninefold lower (6 vs. 55 ng/300,000 PMNs) in PMNs treated with oxyMb (30 μg/mL) compared with PMNs without oxyMb. L-NIO eliminated 100% of the oxyMb-induced decreases in ATP concentration. Just as 30 μg/mL of oxyMb resulted in the highest concentration of NO·, this concentration of oxyMb also produced the most adverse effect in all four apoptotic measures.

A mixed PMN/lymphocyte preparation was used to determine whether the NO· produced by PMNs could affect the plasma membrane integrity of neighboring lymphocytes. PMA-activated and oxyMb (30 μg/mL)-treated PMNs decreased lymphocyte plasma membrane integrity as the ratio of PMNs/lymphocyte increased from 0:1 (76%) to 4:1 (68%) to 10:1 (61%). Preincubation of PMNs with L-NMMA before activation with PMA and oxyMb eliminated the PMN-induced increases in plasma membrane damage in lymphocytes (**Fig. 11**). These results support the premise that NO· originating from PMNs causes cellular damage to neighboring cells.

DISCUSSION

Human PMNs were selected as a model to assess the effect of oxyHb or oxyMb on cells that produce both NO· and $O_2^{·-}$. To mimic the extravascular environment in which activated PMNs release oxidants and cause cellular damage, PMNs were isolated and washed to remove RBCs, RBC ghosts, and their cellular components (e.g., free oxyHb, superoxide dismutase, phospholipids, and catalase). Rejecting PMN preparations containing RBC contamination above 1% and removing visible RBCs and RBC ghosts on top of PMN preparation resulted in a consistently higher yield of NO· from PMA-stimulated and oxyMb-treated PMNs. Because various constituents of RBCs bind NO·, it is conceivable that previous reports of low NO· yields by activated PMNs may be due to RBC contamination [33, 38].

The ability of human PMNs to produce NO· has been controversial. Recently, Wallerath et al. [39] have demonstrated neuronal constitutive NOS in PMNs and inducible NOS in eosinophils. Several methods of determining NO·, including NMMA-inhibitable DCFH oxidation [33], NO-dependent oxidation of oxyHb to metHb [21, 40], and formation of nitrite [41],
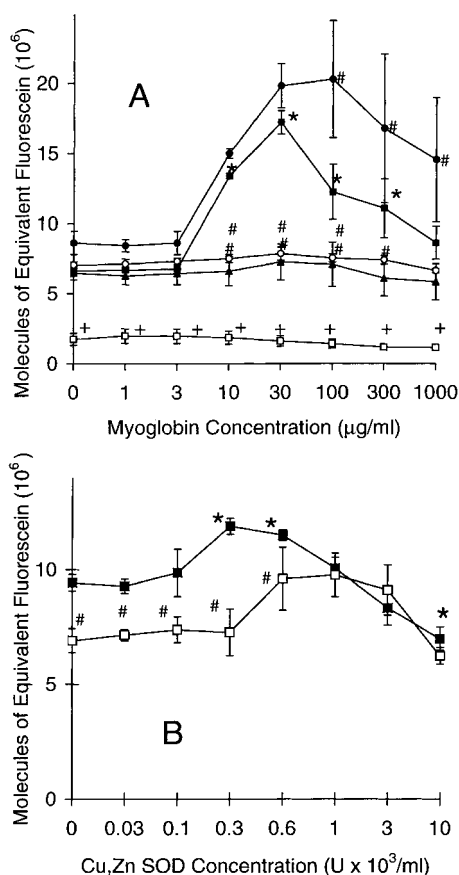
Fig. 8. Effects of NOS inhibitors, antioxidants, and Cu,Zn SOD on oxyMb-induced DCFH oxidation in PMA-stimulated PMNs. (A) PMNs in the absence (■) or presence of L-NIO, 5 mM (▲); L-NMMA, 5 mM (○); glutathione, 25 μM and ascorbate, 25 μM (□) or human erythrocyte Cu,Zn SOD, 30 U (●). Data represent means ± SEM of five to seven experiments. Control vs. concentrations of oxyMb, *$P < 0.05$; oxyMb vs. comparable concentrations of oxyMb + L-NMMA, L-NIO, or SOD, #$P < 0.05$; oxyMb + L-NMMA vs. comparable concentrations of oxyMb + glutathione and ascorbate, +$P < 0.05$. (B) Various concentrations of bovine liver Cu,Zn SOD (30–10,000 U/mL) in the presence oxyMb (30 μg/mL) with (□) and without L-NMMA (■). Data represent means ± SEM of three experiments. OxyMb only vs. Cu, Zn SOD + oxyMb, *$P < 0.05$; without vs. with L-NMMA, #$P < 0.05$.

have reported NO production by PMNs only in the presence of SOD or inhibitors of $O_2^{.-}$ synthesis. Depending on the type and level of PMN stimulation, $O_2^{.-}$ synthesis was 2–50 times NO· levels in these studies [21, 41]. Because activated human PMNs produce both NO and $O_2^{.-}$, which reacts at the rate of diffusion to produce ONOO⁻, it follows that NO· levels will increase as $O_2^{.-}$ levels are attenuated.

Bass et al. [27] established the use of DCFH-DA to measure H_2O_2 production in human PMNs by cytofluorometry. Using this procedure, Rao et al. [33] reported that inhibition of $O_2^{.-}$ production increased DCFH oxidation (i.e., increased DCF fluorescence) in PMNs instead of causing the expected decrease. Their data indicated that the increased DCFH oxidation was the result of reduced scavenging of NO levels by $O_2^{.-}$ and that this increased DCF fluorescence could be attenuated by NMMA. Recently, this strategy of using L-NMMA or *N*-nitro-L-arginine to inhibit NO-induced DCF fluorescence was employed in cytofluorometric assessments of NO· levels in neurons and macrophages [34, 35]. In one of these studies [35], high

levels of oxyHb (650 μg/mL) completely inhibited NO·-induced DCF fluorescence, consistent with our findings. In our application of DCFH-DA in measuring intracellular oxidants, DEANO-derived NO· and H_2O_2 were equally potent oxidants, with equal molar concentrations (10^{-11}–10^{-5} M) inducing similar levels of DCF fluorescence. Because both NO· and H_2O_2 permeate cell membranes, they appear to oxidize DCFH to a similar extent. Although 1 mole of DEANO can theoretically release 2 mol of NO· with a half life of 2.1 min [42] in biological assays, NO· derived from DEANO has been shown to be closer to an equal molar ratio [42, 43]. Higher concentrations of NO· and H_2O_2 (10^{-4} M) resulted in reduced levels of DCF fluorescence that could be explained by leakage of DCF through damaged PMN membranes. Currently, the extent to which intracellular DCFH is directly oxidized by NO· and/or by the NO· oxidation products, nitrogen dioxide ($\cdot NO^2$) or ONOO⁻, is unresolved [44].

DCFH oxidation increased linearly in PMA-activated PMNs as the number (i.e., density) of PMNs increased from 50,000 to 500,000 in a fixed volume. Because the mean cellular DCFH oxidation is determined using a constant number of PMNs (i.e., 10,000), this indicates that oxidants are originating from neighboring PMNs and that intracellular oxidant load increases with PMN density. Bass et al. [27] also observed an increase in intercellular DCFH oxidation in PMNs from chronic granulomatous disease patients as the percentage of PMA-activated PMNs from normal donors increased. PMNs from patients with chronic granulomatous disease activated with PMA do not oxidize DCFH.

Our study shows that oxyMb concentrations produced a concentration-dependent, biphasic effect on DCFH oxidation (i.e., increased NO levels) in unstimulated, LPS and TNF-α-, and PMA-stimulated PMNs. In each of these conditions, increasing concentrations of oxyMb up to 30 μg/mL incrementally increased DCFH oxidation. Higher concentrations of oxyMb resulted in a reduction in DCFH oxidation with the highest concentration of oxyMb (1000 μg/mL) returning DCFH oxidation to control levels. In PMA-stimulated PMNs, oxyHb produced a similar profile but the DCFH oxidation levels were
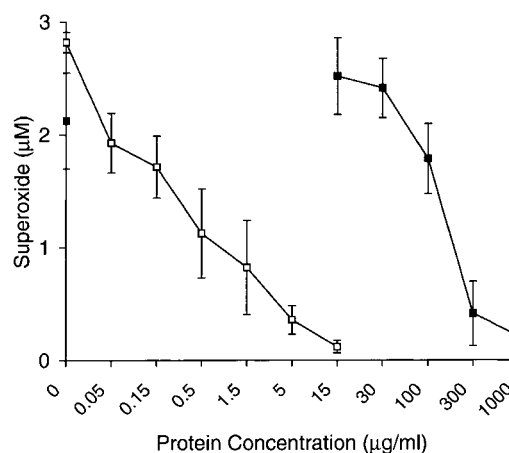

Fig. 9. Concentration-dependent effects of oxyMb (■) or SOD (□) on extracellular $O_2^{.-}$ produced by PMA-stimulated PMNs. Data represent means ± SEM of four experiments.
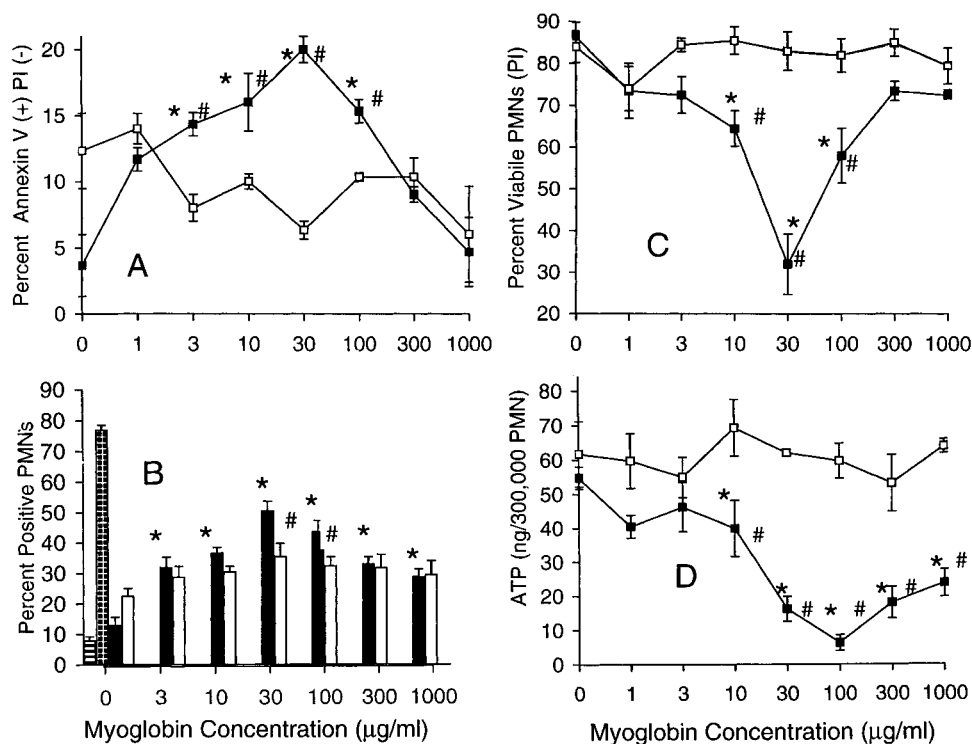
Fig. 10. Concentration-dependent effect of oxyMb on four apoptotic endpoints in PMA-stimulated PMNs. (A) OxyMb effect in the absence (■) or presence of L-NMMA, 5 mM (□) on the phosphatidylserine externalization on PMN's plasma membrane as determined by annexin V binding. (B) OxyMb effect in the absence (■) or presence of L-NMMA, 5 mM (□) on DNA strand breaks in PMNs. Labeling solution without terminal transferase (hatched bar) and with DNase I (cross-hatched bar) were used as negative and positive controls, respectively. (C) OxyMb effect in the absence (■) or presence of L-NMMA, 5 mM (□) on the viability of PMA-stimulated PMNs as determined by intracellular levels of PI. (D) OxyMb effect in the absence (■) or presence of L-NIO, 5 mM (□) on ATP levels in PMA-stimulated PMNs. Data represent means ± SEM of three to seven experiments. Control vs. concentrations of oxyMb, *$P < 0.05$; oxyMb vs. comparable concentrations of oxyMb + L-NMMA, or L-NIO, #$P < 0.05$.

lower compared with equal concentrations of oxyMb (i.e., equimolar with respect to heme). S-nitrosylation of the free sulfhydryl group on oxyHb has been reported to reduce NO· levels compared with oxyMb, which does not contain a free sulfhydryl group [36, 37, 45]. Heme protein effects appear to be dependent on the molar concentration of heme moieties. Because the molecular weights of the subunits of oxyHb and oxyMb are approximately equal, data expressed in concentration (μg/mL) of heme proteins have approximately equal moles of heme. In contrast, concordant amounts of oxyHb encapsulated in a cell membrane (i.e., RBCs) did not increase DCFH oxidation but did reduce DCFH oxidation below that of control values at concentrations above 30 μg/mL. However, sonication of RBCs resulted in increased DCFH oxidation intermediate between intact RBCs and purified oxyHb. The decrease in

Fig. 11. Effect of density of PMA-stimulated PMNs on co-incubated lymphocyte viability in the absence (■) or presence of oxyMb (30 μg/mL; □), or oxyMb (30 μg/mL) + L-NMMA, 5 mM (▤) as determined by intracellular levels of PI. Data represent means ± SEM of six experiments. OxyMb vs. without oxyMb, *$P < 0.05$; oxyMb vs. comparable concentrations of oxyMb + L-NMMA, #$P < 0.05$.

DCFH oxidation (i.e., NO levels) in PMNs incubated with sonicated RBC, compared with comparable amounts of free oxyHb in the sonicated RBCs, could be attributed to other constituents of RBCs such as SOD and catalase (data not shown). The heme protein-induced increases in DCFH oxidation were not due to ferrous iron because equal molar concentrations of Fe^{2+} resulted in a reduction in DCFH oxidation. In PMN preparations without arginine, equal concentrations of heme proteins produced similar DCFH oxidation profiles regardless of the oxidation state of heme iron or chemical modification (i.e., α-α diaspirin cross-linked oxyHb). However, metHb was the only heme protein that did not significantly increase DCFH oxidation.

Activation of PMNs increases oxyHb binding to the PMN's cell membrane with subsequent oxyHb internalization. The binding/internalization of heme proteins into PMNs parallels heme protein-induced increases in NO· levels and cytotoxic effects in PMNs (compare Figs. 4, 5, 6, and 10). Kim et al. [18] have reported that oxyHb binds to PMNs in 30 min (as was true in our experiments) and that oxyHb binding increased several-fold in preparations of PMA-activated PMNs compared with unstimulated PMNs. We have also observed that encapsulated oxyHb (i.e., intact RBC) did not increase NO· levels in activated PMNs compared with sonicated RBCs or a comparable amount of free oxyHb, suggesting that free oxyHb must interact with PMNs to increase NO levels and cytotoxic effects. Kim et al. [18] reported that oxyHb (2 μM; 128 μg/mL) incubated with PMA-activated PMNs reduced extracellular $O_2^{·-}$ levels and PMN viability (trypan blue exclusion) and increased lipid peroxidation of PMNs and survival of extracellular *Escherichia coli*. The effects that heme proteins in different tissue compartments (e.g., extracellular, membrane-bound, and intracellular) have on PMN oxidant levels and cytotoxicity are unknown and merit further study. Therapeutic agents that inhibit heme
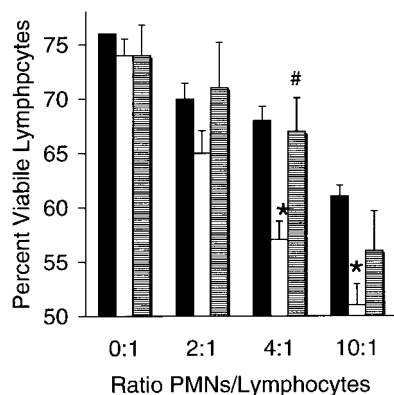
protein binding/internalization into PMNs and other cell types that produce oxidants may have clinical value.

The heme protein-induced increases in DCFH oxidation in PMNs appear to be NO˙ mediated. Two NO˙ synthase inhibitors, L-NMMA, a competitive inhibitor, and L-NIO, a noncompetitive inhibitor, eliminated the oxyMb-induced increase in DCFH oxidation in PMA-stimulated PMNs. In addition, NO˙ levels in PMA-stimulated PMNs were markedly increased by the addition of physiological levels of arginine to HBSS. Moreover, the increase in DCFH oxidation in PMNs paralleled total nitrite concentrations in PMN filtrates. Imrich and Kobzik also observed a direct relationship between DCFH oxidation and nitrite levels [35]. Taken together, these effects indicate that NO˙ is the primary oxidant increasing DCF fluorescence in heme protein-treated PMNs and that a percentage of NO˙ is oxidizing oxyMb to metMb and forming nitrates [36].

We have demonstrated that oxyMb-induced increases in NO˙ levels can be eliminated by physiological levels of ascorbate (25 μM) and glutathione (25 μM), and reduced by extracellular levels of albumin (330 μM; 20 mg/mL). Reduction in NO˙ levels by glutathione or albumin could be the result of S-nitrosylation of an available cysteine residue in each thiol protein [46]. Due to the probability of a larger interstitial space (i.e., extracellular volume) in our preparations as compared to tissues, the antioxidant capacity will likely be greater than *in vivo*. Even during inflammation, when the extracellular volume in tissues may double, the extracellular volume is less than the volume of the PMNs [47]. In our preparations, PMNs are allowed to settle to the bottom of a round-bottom tube containing buffer that is 500 times the volume of the PMNs while being rocked in a water bath.

We propose the following model to account for the bell-shaped, concentration-dependent heme protein effects on intracellular levels of NO˙. Upon activation, NADPH oxidase of PMNs releases the negatively charged $O_2^{˙-}$ to the cell exterior [48] where it interacts with heme proteins. Internalized heme proteins could also interact with $O_2^{˙-}$ produced by PMN vacuoles. Because the reaction of $O_2^{˙-}$ with NO˙ to form $ONOO^-$ is extremely rapid (3.7×10^7 $M^{-1} \cdot s^{-1}$) [22], it is conceivable that heme proteins (3–30 μg/mL) bound to plasma or vacuole membranes bind and/or dismute $O_2^{˙-}$, thereby increasing the NO˙ concentration by limiting the scavenging action of $O_2^{˙-}$. This model is consistent with other reported data showing that PMNs activated with the PMA concentration used in this investigation produced twice the number of moles of $O_2^{˙-}$ as NO˙ [21] and that low concentrations of SOD increase NO˙ levels [18, 49, 50]. Therefore, at low concentrations of heme proteins, NO levels are increased by virtue of the heme binding and/or the dismuting of $O_2^{˙-}$. However, at concentrations of oxyMb above 30 μg/mL, additional heme proteins located at the cell surface, internalized, or in solution, removed NO˙ to a greater extent, thereby abolishing the effect of lowered $O_2^{˙-}$. In experiments conducted at atmospheric oxygen levels, heme proteins are almost fully oxygenated [36]. OxyHb (24×10^6 $M^{-1} \cdot s^{-1}$) or oxyMb (17×10^6 $M^{-1} \cdot s^{-1}$) rapidly reacts with NO to produce metHb or metMb and nitrate [24, 36]. Internalized heme proteins could form the anion, nitrate, in PMNs, thereby reducing extracellular levels of nitrates. We observed that nitrite/nitrate levels in filtrates of PMA-activated PMNs progressively decreased at oxyMb concentrations above 30 μg/mL.

Heme proteins mimicked Cu,Zn-SOD in dismuting extracelluar $O_2^{˙-}$ and in increasing intracellular NO˙ levels. The concentration-dependent profiles for eliminating extracellular $O_2^{˙-}$ were similar for oxyMb and SOD but the oxyMb profile was shifted two orders of magnitude to the right. Also, human erythrocyte (300 U/mL; 800 μg/mL) and bovine liver Cu,Zn-SOD (300–600 U/mL) increased intracellular NO levels in PMA-stimulated PMNs beyond oxyMb treatment only. Like heme proteins, SOD induced a biphasic, concentration-dependent alteration in NO˙ levels, with the highest concentration of SOD reducing fluorescence below control levels. At the lower SOD levels, L-NMMA eliminated the SOD-induced DCF fluorescence, suggesting that SOD increased NO˙ levels and not H_2O_2 levels. These SOD-induced NO˙ levels may be attenuated at higher SOD levels by the formation of *s*-nitrosothiol [51] in a similar manner as in oxyHb [46].

Several reports have shown that the human Cu,Zn-SOD expressed in mice or cell lines potentiates oxidant damage that leads to cellular damage and death. Transgenic mice over-expressing human extracellular superoxide dismutase by five-fold in brain tissues showed a dramatically increased mortality (83%) compared with non-transgenic littermates (33%) on exposure to 6 atm of hyperbaric O_2 for 25 min. The increased mortality was eliminated by either diethyldithiocarbamate, which inhibits SOD, or *N*-nitro-L-arginine, a NOS inhibitor [52]. These transgenic mice also had higher lipid peroxidation and abnormal neuromuscular junctions [53, 54]. Furthermore, the biphasic Cu,Zn-SOD effect was reported in a human hepatoma liver cell line (HepG2) treated with 3-morpholinosynonimine (SIN-1), which releases both NO˙ and $O_2^{˙-}$. Cu,Zn-SOD potentiated SIN-1 cytotoxicity in a concentration-dependent manner up to 100 units of Cu,Zn-SOD/mL but decreased toxicity at higher concentrations [55]. These reports support our contention that compounds dismuting and/or sequestering $O_2^{˙-}$ can increase cellular damage by preventing $O_2^{˙-}$-mediated inactivation of NO˙.

In our study, oxyMb-induced increases in NO˙ levels paralleled increases in several cellular alterations associated with apoptosis in PMNs: phosphatidylserine externalization to the outer surface of the plasma membrane, cell membrane damage, DNA strand breaks, and decreases in cellular ATP levels. In each of the four apoptotic measures, oxyMb-induced NO˙ effects were reversed by NOS inhibitors. Although the effects of oxyHb on these apoptotic measures were not assessed, we would predict similar results.

Externalization of phosphotidylserine from the inner leaflet of the plasma membrane to the outer surface has been reported to be an early marker for apoptosis in human PMNs initiated by several apoptotic agents [56, 57]. OxyMb-induced externalization of phosphatidylserine was observed after 1 h of treatment, before plasma membrane damage was detected by PI. NO˙ effects on membrane integrity could be attributed to direct oxidation of membrane components and/or decreased cellular levels of ATP required for maintaining the cell membrane function and integrity. NO˙ has been reported to inhibit cellular enzymes and macromolecules containing Fe-S clusters or heme

groups, such as mitochondrial aconitase activity, as well as complexes I and II, and cytochrome oxidase of the electron transport chain [58–60]. NO· inhibition of any of these Fe-proteins could reduce ATP synthesis.

NO has also been shown to cause DNA single-strand breaks [61] and DNA deamination [62] and to inhibit DNA synthesis via inhibition of ribonucleotide reductase [63]. These NO·-induced DNA strand breaks activate poly (adenosine 5'-diphosphoribose) synthetase, which further depletes cellular ATP levels [64]. Increasing the level of $O_2^{·-}$, by adding SIN-1 or 12-O-tetradecanoylphorbol-13-acetate (TPA), markedly reduced the level of DNA fragmentation [65]. Similar results were observed in rat PMNs and in a cell-free system where increasing $O_2^{·-}$ levels reduced the N-nitrosation of primary aromatic amines, which was reversed by addition of SOD [49]. Taken together, these results indicate that NO·-induced apoptosis in intact cells is reduced when NO· is scavenged with increasing concentrations of $O_2^{·-}$ and would demonstrate a NO·-regulating role for $O_2^{·-}$.

The concentration-dependent effect of PMA-activated and oxyMb-treated PMNs on the integrity of the plasma membrane of co-incubated lymphocytes reinforces the previous observation that toxic levels of NO· are originating from neighboring PMNs [27]. The ability of L-NMMA to reverse the effects of activated PMNs on lymphocyte membrane integrity further supports this contention.

Elevated levels of free heme proteins have been reported in transfused blood and in the vasculature during numerous pathologies. Hemolysis of RBCs in stored blood increases with time and results in approximately 1% hemolysis after 35 days [9, 10]. OxyMb and oxyHb levels in serum of patients with severe burns frequently reach levels of 3–30 μg/mL [1–3]. Similar levels have been reported in patients with rhabdomyolysis (10 μg/mL) and crush injuries (30 μg/mL) [3, 4]. Lower levels (1–3 μg/mL) have been reported in serum of patients after myocardial infarction [6]. However, higher myoglobin levels would be predicted in areas surrounding damaged myocardial and skeletal muscle tissue. In this study, levels of heme proteins found in the vasculature during various pathologies increased intracellular levels of NO· and cytotoxicity in activated PMNs and neighboring lymphocytes.

The role of hemolysis of RBCs in transfused blood in the predisposition to infections, multiple organ failure, and recurrence of cancer has been established but is not understood [13, 14, 66]. Lysed RBCs not only release Hb but also Cu,Zn SOD, catalase, phosphatidyserine, and phosphatidylethanolamine from the inner leaflet of the plasma membrane. Phosphatidyserine and phosphatidylethanolamine-treated macrophages increased oxidant levels and lipid peroxides in the incubation media [67]. Plasma Hb in transfused whole blood has been measured as high as 440 μg/mL and as high as 5,780 μg/mL in red cell concentrates. At these levels of free Hb, it is evident that transfusions could decrease extracellular $O_2^{·-}$ levels around cells that produce NO· and $O_2^{·-}$, thereby increasing the potential for NO-mediated cytoxic effects on neighboring cells. Our proposed model of heme protein-induced damage to host defense and other cells may explain transfusion-induced increases in infection, tissue damage, and recurrence of cancer.

In summary, disease processes that release heme proteins may result in the binding of heme protein to activated PMNs and cause NO·-induced damage to tissue at proximal sites. The amount of tissue damage will be contingent on the type and concentration of oxidants interacting with heme proteins and the levels of antioxidants in the tissue. The described mechanism of the cytotoxic effects of heme proteins suggests that therapeutic interventions should focus on strategies of both reducing NO· levels in tissues and levels of free heme proteins. The first strategy could focus on inhibiting PMN migration into damaged tissues, inhibiting NO· production, and maintaining adequate antioxidants in the tissues to scavenge oxidants. The second strategy could focus on minimizing damage to cells that release heme proteins, removing heme proteins from serum by plasmapheresis and binding to haptoglobin, reduce binding of heme proteins to cells that produce oxidants, and oxidizing the heme protein to bilirubin by inducing the synthesis of heme oxygenase [8, 17, 68]. Furthermore, our results indicate the potential adverse effects of using cell-free oxyHb substitutes (e.g., α-α diaspirin cross-linked Hb) in patients with an activated immune system. This mechanism may explain the increased mortality in trauma patients transfused with cross-linked Hb [15]. Finally, our studies may explain the increase in severity of risks related to duration of blood storage and provide support for the study of improved preservation of RBC [66, 69] used to treat patients with an activated immune system.

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